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Inventor(s): REHBERGER et al.
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RULE 132 DECLARATION OF THOMAS G. REHBERGER

I, THOMAS G. REHBERGER, the undersigned Declarant, do hereby state and declare that:

1. I am intimately familiar with the invention described and claimed in the above-identified patent application. I am a co-inventor of the invention described and claimed herein.

2. From 1993 to the present, I have been a Partner and Vice-President of Agtech Products, Inc. I received my B.S. in Biological Sciences from Iowa State University in 1982. I received my Ph.D. in Food Microbiology from Iowa State University in 1988. My *Curriculum vitae* is attached hereto as Exhibit A and incorporated herein by reference. I am one skilled in the art of the instant patent application.

3. To demonstrate that the strains recited in the method claims of the above-identified patent application are different from the strains disclosed in U.S. Patent No. 6,455,063 to Rehberger, et al., U.S. Patent No. 6,120,810 to Rehberger, et al., and U.S. Patent No. 5,139,777 to Ott, et al., the following experiments were conducted with my participation and under my direct control and supervision.

4. Strains Tested: The following microorganisms of the *Propionibacterium* genus were tested: P63 (disclosed in U.S. Patent No. 6,455,063 to Rehberger, et al.) and P5 (disclosed in U.S. Patent No. 6,120,810 to Rehberger, et al.).

a. The strain Hh-GYOKI-1-123Sz (disclosed in U.S. Patent No. 5,139,777 to Ott, et al., Accession No. NCAIM B(P) 000287 of the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary) was *not* tested due to its unavailability from the National Collection in Budapest. I have made several attempts to obtain the Hh-GYOKI-1-123Sz strain (disclosed in U.S. Patent No. 5,139,777 to Ott, et al.), Accession No. NCAIM B(P) 000287 from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary (hereinafter "the Collection"). Attempts were made by email, the Collection's web site, and telephone.

b. On January 3, 2004, I emailed Dr. Judit Tomai-Lehoczki of the Collection to ask her for the Hh-GYOKI-1-123Sz strain. A copy of my email is attached as Exhibit B and incorporated herein by reference. Dr. Tomai-Lehoczki did not respond to my January 3 email. I then emailed Dr. Tomai-Lehoczki again, this time in German, by using a web-based translator in which I translated the text of my initial email into German. Again, I received no reply from Dr. Tomai-Lehoczki. After showing the web-based translated email to an employee who is fluent in German, I again emailed Dr. Tomai-Lehoczki, this time with the assistance of this employee. Again, there was no reply. I then emailed Dr. Tomai-Lehoczki two more times, both in English, and received no reply. I stopped trying to contact Dr. Tomai-Lehoczki the week of January 26, 2004, because at that time I knew that even if I received the requested culture from her, there would not be enough time to grow it and run the required tests before the deadline to respond to the outstanding Office Action.

c. In addition, on January 9, 2003, I viewed the list of available strains from the Collection via the web. A printout of the list is attached hereto as Exhibit C and is incorporated herein by reference. As can be seen from the list, only two *Propionibacterium* strains are listed, and there is no listing of the above-mentioned strain that was referenced in the patent to Ott et al.

d. I also called the Collection and talked to an employee of the Collection. The employee seemed to understand my request. I told them which strain I needed and that I also needed a fax with instructions on how to obtain the strain. However, I never received the requested fax or the requested strain.

e. In sum, although multiple tactics and multiple attempts were made to obtain the above-referenced strain, this was not possible.

f. The two other strains disclosed and deposited by Ott, et al. in the '777 patent were not tested because neither of these strains are of the genus Propionibacterium: Hh-GYOKI-2-14Ab is of the *Veillonella* genus, (Ott, et al., col. 5, lines 6-7) and Hh-GYOKI-3-81Me is of the *Bifidobacteria* genus. (Ott, et al., col. 5, lines 17-19). One additional strain of the *Propionibacterium* genus is mentioned in the Ott, et al. patent: Hh-GYOKI-48a, which is said to originate from Hh-GYOKI-1-123. (Ott, et al. patent; col. 4, lines 61-63). However, this strain was not deposited at, and was not available from the National Collection in Budapest (see Exhibit C for list of strains that are available). Furthermore, the specifics on how Hh-GYOKI-48a was derived from Hh-GYOKI-1-123 are not disclosed in the Ott, et al. patent. Therefore, the Hh-GYOKI-48a strain was not tested.

5. Pulsed-Field Gel Electrophoresis and Randomly Amplified Polymorphic DNA (RAPD) Analyses:

a. Materials and Methods: Pulsed Field Gel Electrophoresis:

Bacterial strains and culture conditions. All *Propionibacterium* strains were obtained from the culture collection at Agtech Products, Inc. and routinely grown in sodium lactate broth at 32°C.

Preparation of intact genomic DNA in agarose beads. Intact genomic DNA from each strain was isolated from cells embedded in agarose beads, generally by the method described in the patent application on p. 10, lines 7-10 and Example 1. A difference from the method described in the patent application is that two lysis procedures were used. For lysis procedure 1, cultures were grown to mid-log state in NLB. For lysis procedure 2, cultures were grown to mid-log state in NLB supplemented with 2% glycine, which weakens cell walls, thereby improving DNA isolation from the cells. Both sets of cultures were then washed with sterile distilled water, harvested by centrifugation (9000 g for 10 min), and resuspended to one-tenth the original volume in ET buffer (50mM EDTA, 1mM Tris-HCI, pH 8.0). The cell suspension was warmed to 45°C and mixed with an equal volume of 1% low-melting-point agarose. The mixture was poured into a 10 ml syringe and slowly injected into a 6-foot length of tygon® tubing (1/16" ID x 1/8" OD). Once the agarose solidified, the solidified strand was flushed out of the tubing with 10 ml of 10X ET buffer and collected in a 25 ml centrifuge tube. The strand was cut into smaller pieces (2-3 mm) with a plastic spatula, and then harvested by centrifugation (4000 *g* for 10 min at 4°C). The beads were resuspended in 10 ml of 10X ET buffer containing 20 mg/ml of lysozyme

and were incubated at 32°C for 2 h with slow shaking (120 rpm) to digest the cell wall material. After incubation, the beads were harvested by centrifugation (4000 *g* for 10 min) and resuspended in 10 ml of lysis buffer (10X ET buffer containing 100 µg/ml of proteinase K and 1% [w/v] Sarkosyl). The beads were incubated at 55°C overnight with slow shaking (120 rpm) to lyse the cells and release the genomic DNA. After cellular lysis, the beads were harvested by centrifugation (4000 *g* for 10 min), resuspended in 10 ml of 1mM phenylmethylsulfonyl fluoride, and incubated at room temperature for 2 h with slow shaking (120 rpm). The beads containing the purified DNA were washed three times in TE buffer (10 mM Tris-HCI, 1mM EDTA –Na_2, pH 7.5), resuspended in 5 ml of TE buffer, and stored at 4°C until restriction endonuclease digestion.

Restriction endonuclease digestion of DNA and pulsed-field gel electrophoresis. Agarose beads (90 µl per digest) were equilibrated in 1X restriction endonuclease digestion buffer at 4°C for 1 h. After equilibration, 10-15 units of the restriction enzyme were added, and the DNA was digested at the recommended temperature for 4-6 h. After digestion, the agarose beads were melted at 65°C for 5 min and immediately loaded into the wells of a 0.8% agarose gel. The melted beads were allowed to solidify, and the wells were capped with molten agarose before the gel was placed in the CHEF-DR® III apparatus. The gel was prepared and run with 0.5X TBE buffer. The program was run at 14°C for 19 hours and 44 minutes with an initial switch time of 1.47 seconds and a final switch time of 29.08 seconds. The angle was 120° and the voltage gradient was set at 6 V/cm. The gel was stained for 30 minutes in 0.5 µg/ml Ethidium bromide solution and destained for 2 hours

in 500 ml Milli-Q water. The DNA was visualized by UV transillumination and the image was captured using the Syngene BioImaging system.

b. Materials and Methods: Randomly Amplified Polymorphic DNA (RAPD) PCR:

PCR amplifications were carried out in 25 µl volumes. RAPD analysis was performed using Ready-to-Go RAPD Beads (Amersham Biosciences, Buckinghamshire, England) containing two thermostable polymerases (AmpliTAQ and the Stoffel fragment), dNTPs and buffer in a room temperature stable bead. Each bead was reconstituted with 16 µl of sterile dH_2O, 5 µl of primer (5 pmol/µl), and 4 µl genomic DNA. Two primers, each in separate reactions, were used for RAPD PCR analysis, Primer 1: 5'-d[GGTGCGGGAA]-3' and Primer 2: 5'-d[GTTTCGCTCC]-3'. Amplification for RAPD analysis was performed in a GeneAmp PCR System Thermal Cycler (Applied Biosystems) programmed for a pre-incubation of 95°C for 4 min., followed by 45 cycles of: 94°C for 1 min., 36°C for 1 min., and 72°C for 2 min. PCR products were identified on a 1% agarose gel (Bio-Rad) and visualized by UV transillumination after staining in ethidium bromide solution. Gel images were captured using the Syngene BioImaging system.

c. Results: Pulsed Field Gel Electrophoresis:

Isolation of the intact chromosomal DNA using solid state techniques followed by restriction endonuclease digestion coupled with pulsed field agarose gel electrophoresis allows for strain comparisons using the DNA restriction fragments produced. Since the restriction endonucleases cut

sites are determined by a know recognition sequence, the number of cut sites in a chromosome and the resulting number of DNA fragments represent the number times the cut recognition site occurs in the chromosome. The size or molecular weight of these fragments is related to the distance these cut recognition sites are from each other in the chromosome. The number of fragments and their molecular weight are the basis of determining the relatedness of strains using this technique.

Figure 1, attached hereto as Exhibit D and incorporated herein by reference, is a pulsed field gel of the chromosomal DNA from strain P5, P63 and P169 digested with the restriction enzyme Xba I. **Lane 1** is a lambda ladder or standard; **lane 2**, strain P5 lysis procedure 1 (without glycine) Xba I digested; **lane 3**, strain P5 lysis procedure 2 Xba I digested; **lane 4**, P169 lysis procedure 1- Xba I digested; **lane 5** strain P169 lysis procedure 2 (with glycine) - Xba I digested; **lane 6**, strain P169 lysis procedure 2- Xba I digested; **lane 7**, lambda standard; **lane 8**, strain P63 lysis procedure 1- Xba I digested.

The results of the Xba I digested chromosomal DNA indicate each strain produced a unique set of Xba I fragments. Therefore, each strain is unique and strain P169, a representative of a strain having a group I profile produced by *Xba* I digests of genomic DNA as shown in Figures 1-2 and Table 3 of the above-identified patent application, is not identical to strain P5 or P63. Although the pattern for P63 was faint in this gel, the fragments did not co-migrate with the fragments of P5 or P169. To ensure these results were accurate, another DNA fingerprinting method was employed, randomly amplified polymorphic DNA PCR analysis.

d. Results: RAPD PCR Analysis:

RAPD PCR analysis of strains P5, P63 and P169 attached hereto as Exhibit E and incorporated herein by reference are shown in Figure 2 herein by reference. In RAPD PCR analysis a primer of arbitrary sequence generally 10 base pairs long is added to the PCR reaction and will anneal to the DNA template (propionibacteria strain) and prime the DNA polymerase to make a complementary copy of one of the DNA strands. In the beginning of the second cycle of the PCR reaction, the DNA molecule is denatured to two single strands. The primer anneals to the single strand made in the first cycle and will prime the DNA polymerase reaction in the opposite direction leading to a defined DNA fragment. This fragment's molecular weight is determined by the location of the homology of the primer to both strands of the original DNA molecule and therefore represents a strain specific fragment. The total collection of these fragments (number and size) represent the homologous locations of the primer to the original DNA strands and therefore serves as a DNA fingerprint of the original strain.

Two different primers were used to determine the relatedness of the three propionibacteria strains in question, i.e., P5, P63 and P169. The results indicate that primer 1 produced six major DNA fragments ranging in weight from 100-1000 base pairs for strain P5 (lane 2). Primer 1 produced five major DNA fragments ranging in weight from 300-1500 for strain P63 (lane 3) and one major fragment of about 450 base pairs for strain P169 (lane 4).

The DNA finger print produced by primer 2 is shown in lanes 6-8 of Figure 2. Primer 2 produced four major DNA fragments ranging in weight from 600-1200 base pairs for strain P5 and 6 or more fragments ranging in weight from 400-1100 base pairs for strain P63. Primer 2 produced a unique pattern of four fragments ranging in weight from 350-900 base pairs.

Collectively, these results indicate that DNA fragment pattern produced by primer 1 and primer 2 for strain P169 are different than the DNA fragment patterns produced for these same primers for strain P5 and P63. Therefore, strain P169 is different than strain P5 and strain P63.

e. Conclusion of PFGE and RAPD PCR Analyses:

The results of pulsed field gel electrophoresis and RAPD PCR indicate that strain P169, a representative of a strain having a group I profile produced by *Xba* I digests of genomic DNA as shown in Figures 1-2 and Table 3 of the above-identified patent application, is not identical to strain P5 or P63.

6. Biochemical Analysis:

a. Propionic Acid Production:

High pressure liquid chromatography (HPLC) analysis was used to quantify the production of propionic acid from strains P5 and P63 to compare to the levels reported with strain P169. The strains were grown in sodium lactate broth at 32°C. Samples were collected at 24 and 72 hours and analyzed for propionic acid using HPLC. It should be noted that the HPLC data shown in the patent application is from 48 hours.

However, an increase in propionate would be expected over time, as can be seen by comparing the 24 hour and 72 hour levels. HPLC chromatograms are attached as Exhibit F and are incorporated herein by reference. The results are shown in Table 1 below. It should be noted that in Example 1 of the patent application, the propionate levels were measured at 48 hours, whereas in the new materials, levels were measured at 24 and 72 hours. An increase in propionate levels would be expected over time, as can be seen by comparing the 24 hour levels to the 72 hour levels.

Table 1

Strain	Time	Propionic acid
P5	24 h	0.138%
	72 h	0.403%
P63	24 h	0.075%
	72 h	0.316%

These results indicate that strains P5 and P63 do not produce the at least 0.9% level of propionic acid in sodium lactate broth as required by claim 49 and reported for strain P169.

No data are available for the percent propionate produced in rumen fluid for strains P5 and P63. Claim 3 requires the feeding of a microorganism of the genus *Propionibacterium*, which has the characteristic of producing at least 0.2% (vol/vol/) propionate in rumen fluid (in vitro). However, one skilled in the art would understand that if strains P5 and P63 could not

produce the required at least 0.9% (vol/vol) propionate in the *sodium lactate broth*, it would be completely unexpected that these strains could produce the required level in *rumen fluid* because the sodium lactate broth is a far superior medium compared to rumen fluid. Thus P5 and P63 would not grow as well in rumen fluid and produce the required at least 0.2% (vol/vol) propionate in rumen fluid.

b. Conclusion of Biochemical Analysis:

In addition, the propionic acid level produced by strain P169, a representative strain of the genus *Propionibacterium* that (1) produces at least 0.9% (vol/vol) propionate in sodium lactate broth and (2) produces at least 0.2% (vol/vol) propionate in rumen fluid (in vitro) produced a much higher than propionic acid level the propionic acid level produced by strain P5 and P63, both of which did not produce at least 0.9% (vol/vol) propionate in sodium lactate broth.

7. Enablement: Claims Requiring a Group 1 Profile:

The claims of the above-reference patent application that require a group I profile produced by *Xba* I digests of genomic DNA as shown in Figures 1-2 and Table 3 are sufficiently enabled as of the filing of the instant patent application to allow one skilled in the art to make and use the invention without undue experimentation. The application specifically discloses that the microorganisms of the invention were isolated from the rumen of fistulated ruminants and were selected from the genus *Propionibacterium*. (page 9, lines 14-16). The application explains how the bacteria were isolated, grown, and the strains determined based on biochemical tests and carbohydrate fermentation patterns that are known in the art. (page 9, lines

17-21). The application then details how the intact genomic DNA from the isolates was evaluated. Pulsed-field gel electrophoresis analysis of genomic DNA identified 13 distinct *Xba* I fragment patterns. (p. 10, lines 7-10). The isolates were then tested for volatile fatty acid, and the isolates that produced the highest amount of propionate under conditions similar to the rumen were selected for animal testing. (page 10, lines 12-15). Identification of the propionibacteria isolates, genomic DNA analysis, and volatile fatty acid production is described in further detail in Example 1. These studies showed that strains P169, P170, P179, P195, and P261 were the highest propionate producing strains and that all of these strains had a group I genotype. Animal studies using strain P169 are then detailed in Example 2. A second study of strain P169 in ruminants is described in Example 3. In addition, preferred amounts of bacteria to be fed, conditions of the bacteria, e.g., freeze-dried, and timing of the feeding are disclosed in the application. Thus, others skilled in the art could follow the steps detailed in the patent application for isolating bacteria having a group I profile and carry out the steps of the method claims of the above-captioned patent application that require such bacteria without undue experimentation.

8. Enablement: Claims Requiring Specific Propionate Production:

The claims of the above-reference patent application that require a microorganism of the genus *Propionibacterium* having the characteristics of (1) producing at least 0.9% (vol/vol) propionate in sodium lactate broth, and (2) producing at least 0.2% (vol/vol) propionate in rumen fluid (in vitro) are sufficiently enabled as of the filing of the instant patent application to allow one skilled in the art to make and use the invention

without undue experimentation. As noted above, the application specifically discloses that the microorganisms of the invention were isolated from the rumen of fistulated ruminants and were selected from the genus *Propionibacterium*. Multiple collections of ruminal fluid were obtained over a period of time. (page 9, lines 14-16). Page 18, lines 7-16 of the application details how the percent of proprionate in both sodium lactate broth and in rumen fluid (in vitro) are determined. Thus, others skilled in the art could follow the steps detailed in the patent application for isolating bacteria having the characteristics of (1) producing at least 0.9% (vol/vol) propionate in sodium lactate broth, and (2) producing at least 0.2% (vol/vol) propionate in rumen fluid (in vitro) and carry out the steps of the method claims of the above-captioned patent application that require such bacteria without undue experimentation.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, sunder Section 1002 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Rule 132 Declaration is directed.

Dated: 2/18/04

Thomas Rehberger

THOMAS G. REHBERGER